0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(6):2220–2228

doi: 10.1210/en.2002-0135

The Role of a Conserved Region of the Second Intracellular Loop in AT_1 Angiotensin Receptor Activation and Signaling

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The pleiotropic actions of angiotensin II are mediated by the primarily G_q protein-coupled type 1 angiotensin (AT_1) receptor. In this study a mutational analysis of the function of the conserved DRYXXV/IXXPL domain in the second intracellular loop of the rat AT_{1A} receptor was performed in COS7 cells. Alanine substitution studies showed that single replacement of the highly conserved Asp^{125} and Arg^{126}, but not Tyr^{127}, moderately impaired angiotensin II-induced inositol phosphate signaling. However, concomitant substitution of both Asp^{125} and Arg^{126} caused marked reduction of both inositol phosphate signaling and receptor internalization. Alanine scanning of the adjacent residues showed that substitution of Ile^{130}, His^{132}, and Pro^{133} reduced agonist-induced inositol phosphate signal generation, whereas mutations of Met^{134} also impaired receptor internalization. Expression of the D125A mutant AT_{1A} receptor in COS7 cells endowed the receptor with moderate constitutive activity, as indicated by its enhanced basal Elk1 promoter activity and inositol phosphate response to partial agonists. Angiotensin II- induced stimulation of the Elk1 promoter showed parallel impairment with inositol phosphate signal generation in receptors containing mutations in this region of the AT_{1A} receptor. These data confirm that Ca^{2+} signal generation is required for the nuclear effects of angiotensin II-induced ERK activation. They are also consistent with the role of the conserved DRY sequence of the AT_{1A} receptor in receptor activation, and of Asp^{125} in constraining the receptor in its inactive conformation. Furthermore, in the cytoplasmic helical extension of the third helix, an apolar surface that includes Ile^{130} and Met^{134} appears to have a direct role in G protein coupling. (*Endocrinology* 144: 2220–2228, 2003)

THE TYPE 1 (AT_1) angiotensin receptor is a heptahelical, G protein-coupled receptor (GPCR) that mediates the known physiological actions of the octapeptide hormone, angiotensin II (Ang II; Refs. 1 and 2). Binding of Ang II to the AT_1 receptor leads to stimulation of phospholipase C and inositol phosphate/Ca^{2+} signaling via activation of $G_{q/11}$ proteins (2, 3). The AT_1 receptor can also couple to other G proteins, including G_i, and activates intracellular signaling pathways that stimulate growth responses via activation of tyrosine kinases and small GTP-binding proteins (4, 5). Ang II also causes phosphorylation of the receptor, which predominantly occurs at its cytoplasmic tail (6–8) and regulates agonist-induced internalization and desensitization of the receptor (9, 10).

Current structural models of GPCRs based on the high-resolution crystal structure of bovine rhodopsin (11–13) have confirmed the seven transmembrane nature of GPCRs and indicate that the membrane proximal part of the cytoplasmic tail forms an eighth helix. Although such models are relevant to the resting conformations of many GPCRs, they do not address the structural changes related to the agonist-induced activation of these receptors. It is generally believed that the highly conserved amino acid residues and motifs found in most GPCRs have critical roles in the mechanism of receptor activation. One of the highly conserved sequences is the almost universal DRYXXV/IXXPL motif, which is present in the second intracellular loop of GPCRs of the rhodopsin subfamily. The presence of a bulky hydrophobic amino acid (Leu, Ile, Val, Met, or Phe) at the C-terminal end of this motif is highly conserved. Mutations of the highly conserved and negatively charged aspartate (or glutamate) residue of the DRY sequence have been shown to increase constitutive receptor activity in several GPCRs (14–21). It has been suggested that agonist-induced activation of GPCRs involves protonation of the conserved aspartate residue (16, 22, 23). The crystal structure of the inactive conformation of rhodopsin supports the proposed role of the conserved Glu/Asp residue in stabilizing the inactive receptor conformation (11, 12). The arginine member of the DRY sequence is conserved throughout the rhodopsin subfamily of GPCRs and appears to be a key residue for signal transduction because its mutation causes impaired signal transduction in many receptors (16, 20, 24–27). The conserved arginine in rhodopsin has been shown to interact directly with the G protein to catalyze GDP release (14). However, the DRY sequence is not predicted to be part of the α_{1b}-adrenergic receptor-G_q interface (28), and

Abbreviations: Ang II, Angiotensin II; AT_1 receptor, type 1 angiotensin receptor; DPBS, Dulbecco's PBS; GPCR, G protein-coupled receptor; $InsP_2$, inositol-bisphosphate; $InsP_3$, inositol-trisphosphate; TM3, third transmembrane domain.

the exact function of the conserved arginine in the process leading to G protein activation remains unknown.

The amino acids of IL-2 that are C-terminal to the conserved DRY sequence are believed to serve as a switch that enables G protein coupling (29). In the M5 muscarinic receptor, mutations on one side of this helical region cause constitutive activation, whereas residues on the opposite side of the helix are required for receptor activation (29). The functional importance of the conserved apolar residue, which is in most cases leucine but in the AT_{1A} receptor is methionine, at the C-terminal end of the DRYXXV/IXXPL motif was also reported for several GPCRs (13, 30–33).

Most studies on the role of the conserved DRYXXV/IXXPL motif during GPCR activation have analyzed rhodopsin and aminergic GPCRs, and much less is known about the mechanism of activation of peptidergic receptors. Studies on the AT_1 angiotensin receptor (Fig. 1) have suggested that the DRY sequence of the DRYXXV/IXXPL motif is required for receptor activation (34, 35) and histidine substitutions of the adjacent sequence (from Tyr^{127}-Ile^{130}) interfere with agonist-induced signal generation (36). In the present study, alanine-scanning mutagenesis of the highly conserved DRY-LAIVHPM motif in the second intracellular loop of the AT_1 receptor was performed to analyze the role of this region in its agonist-induced conformational change, G protein activation, and internalization.

Materials and Methods

Materials

Culture media were from Biofluids (Rockville, MD) or Invitrogen Corp. (Carlsbad, CA). Lipofectamine was from Invitrogen. ^{125}I-Ang II and ^{125}I-[Sar^1, Ile^8]Ang II were obtained from Covance Laboratories, Inc. (Vienna, VA) or NEN Life Science Products (Boston, MA), and [^{3}H]inositol was from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals and reagents were from Sigma-Aldrich Corp. (St. Louis, MO) unless otherwise stated.

Mutagenesis and expression of the rat smooth muscle AT_{1A} receptor cDNA

The cDNA of the rat vascular smooth muscle AT_{1A} receptor was donated by Dr. K. E. Bernstein (Emory University, Atlanta, GA). Mutations in the rat AT_{1A} receptor were performed with the Mutagene kit (Bio-Rad Laboratories, Inc., Hercules, CA). Sequences of mutant colonies were verified by dideoxy sequencing using Sequenase II (Amersham-U.S.B., Cleveland, OH). COS7 cells were maintained as described previously (37). Plasmids containing the wild-type and mutant receptor cDNAs were transiently expressed in COS7 cells using Lipofectamine as previously described (6).

[Sar^1,Ile^8]Ang II binding to intact cells

To determine the surface expression level and structural integrity of the mutant receptors, the number and affinity of Ang II-binding sites were determined 48 h after transfection. For this purpose, cells were incubated with ^{125}I-[Sar^1,Ile^8]Ang II (0.05–0.1 μCi/sample) and increasing concentrations of unlabeled [Sar^1,Ile^8]Ang II in DMEM containing 25 mM HEPES (pH 7.4) for 6 h at 4 C. The cells were then washed twice with ice-cold Dulbecco's PBS (DPBS) and the receptor-bound radioactivity was measured by γ-spectrometry after solubilization with 0.5 M NaOH/0.05% sodium dodecyl sulfate. The radioligand-binding inhibition curves were analyzed with the LIGAND computer program using a one-site model (38).

Inositol phosphate measurements

The culture medium was replaced 24 h after transfection with 0.5 ml inositol-free DMEM containing 1 g/liter BSA, 10–20 μCi/ml [^{3}H]inositol, 2.5% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin as described earlier (37). Twenty-four hours later the cells were washed twice, incubated in inositol-free DMEM containing 25 mM HEPES (pH 7.4) and 10 mM LiCl for 30 min at 37 C, and stimulated with 30 nM to 1 μM Ang II for 20 min. Inositol phosphates were extracted as previously described (37), and after neutralization the samples were applied to an AG1 × 8 column (Bio-Rad Laboratories, Inc.). The columns were washed three times with 3 ml water and twice with 3 ml 0.2 M ammonium-formate in 0.1 M formic acid to remove inositol and inositol monophosphates. After these washing steps, the combined $InsP_2$ (inositol biphosphate) + $InsP_3$ (inositol triphosphate) fractions were eluted with two 3-ml aliquots of 1 M ammonium formate in 0.1 M formic acid, and radioactivities were determined by liquid scintillation counting.

Receptor internalization in transiently transfected COS-7 cells

Before each experiment the culture medium was replaced by DMEM containing 1 g/liter BSA and 25 mM HEPES (pH 7.4). To determine the internalization kinetics of the wild-type and mutant AT_1 receptors, ^{125}I-Ang II (0.05–0.1 μCi) was added in the same medium, and the cells were incubated at 37 C for the indicated times. Incubation was terminated by placing the cells on ice and rapidly washing them twice with ice-cold

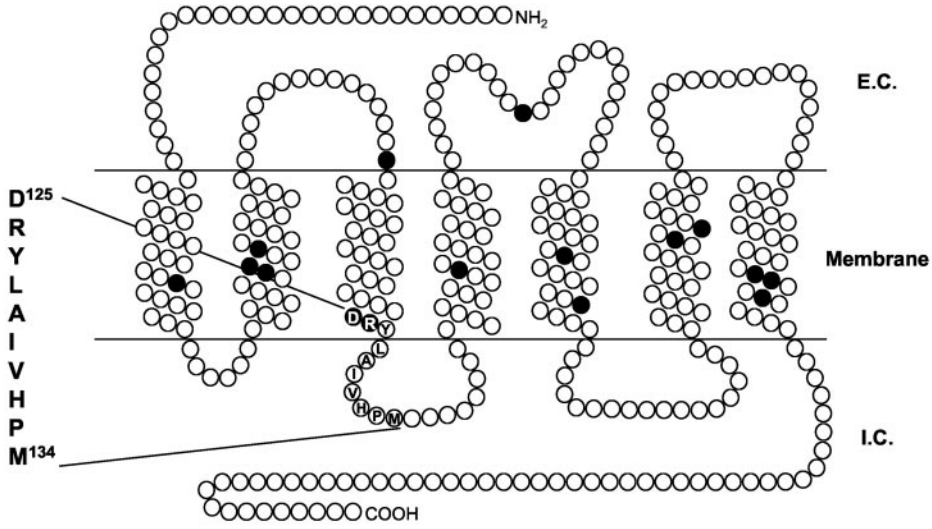

FIG. 1. Structure of the rat AT_{1A} receptor. The positions of the helices, and those of the most conserved residues (*shown in black background*), are based on a comparative study and modeling of more than 200 GPCRs (52). The indicated amino acids were investigated in this study. AT_{1A} receptor mutants are labeled with the *one-letter code* and the position of the exchanged amino acid.

DPBS. Then the cells were incubated for 10 min in 0.5 ml acid wash solution (150 mM NaCl, 50 mM acetic acid) to remove the surface-bound radioligand. The supernatant containing the acid-released radioactivity was collected, and the cells were treated with 0.5 M NaOH and 0.05% sodium dodecyl sulfate to solubilize the acid-resistant (internalized) radioactivity. Radioactivity was measured by γ-spectrometry and the percent internalization for each individual point was calculated from the ratio of the acid-resistant binding to the total (acid-resistant + acid-released) binding. From these data the endocytotic rate constants were calculated as described previously (39).

Measurement of Elk1-regulated luciferase activity

Ang II-induced ERK activation was determined by measuring activation of the Elk1 transcription factor using the PathDetect Elk1 *trans*-reporting system (Stratagene, La Jolla, CA). COS7 cells were cotransfected using Lipofectamine (16 μg/ml) in 24-well plates in 0.4 ml OPTI-MEM per well with the mutant or wild-type AT_{1A} receptors (0.5 μg per sample) and plasmids that encode the activation domain of Elk1 fused to the yeast GAL4 DNA-binding domain (pFA2-Elk1, 5 ng per sample) and firefly luciferase with a synthetic promoter with five tandem repeats of the yeast GAL4-binding sites (pFR-Luc, 100 ng per sample) for 6 h. After transfection, the cells were incubated in DMEM containing 10% fetal calf serum for 24 h, followed by incubation in DMEM containing 1% fetal calf serum overnight. To determine luciferase activity, the cells were incubated in the absence or presence of 1 μM Ang II in serum-free DMEM for 5 h. After removal of the media, the cells were washed twice with 1 ml ice-cold PBS, cell lysates were prepared, and the luciferase activities of the samples were determined in a Berthold LB 953 luminometer using a luciferase assay kit (Promega Corp., Madison, WI) according to the manufacturer's instructions.

Statistical analysis

All data are presented as means ± SEM. Differences between groups were analyzed by *t* test and ANOVA combined with Tukey HSD (honestly significant difference) or Scheffé's multiple comparison test using the software STATISTICA (StatSoft Inc., Tulsa, OK). The value of *P* less than 0.05 was considered significant.

Results

Inositol phosphate responses of $D^{125}R^{126}Y^{127}$-mutant AT_{1A} receptors

The role of the highly conserved $D^{125}R^{126}Y^{127}$ triplet in the function of the AT_1 receptor was analyzed by replacing each amino acid with alanine (Fig. 1). The ability of the mutant receptors to mediate inositol phosphate signal generation was evaluated in transiently transfected COS7 cells prelabeled with [^{3}H]inositol for 24 h. The accumulation of radioactivity in the combined $InsP_2$ and $InsP_3$ fractions was measured after stimulation of the cells with a maximally effective concentration of Ang II (1 μM) for 20 min in the presence of LiCl. Alanine substitutions of the amino acids of the DRY sequence consistently reduced the amplitude of the inositol phosphate response (Fig. 2A). However, the R126A and Y127A mutants also had lower expression levels, compared with the wild-type AT_{1A} receptor (Table 1). We demonstrated in previous studies that the inositol phosphate response in this system is proportional to the receptor expression level (37, 40). On the basis of this observation, no major impairment of the inositol phosphate responses of the D125A, R126A, and Y127A mutants was observed. However, the EC_{50} values of inositol phosphate dose-response curves of the D125A (5.7 ± 1.7 nM, n = 3) and R126A (6.2 ± 2.4 nM, n = 3) mutant AT_{1A} receptors, but not the Y127A (2.9 ± 0.2 nM, n = 4) mutant receptor, showed significant ($P < 0.05$) increases compared with that of the wild-type receptor (1.6 ± 0.3 nM, n = 4), indicating that the G protein coupling of D125A and R126A mutant receptors is moderately impaired. Further analysis of the function of the DRY sequence revealed that alanine replacements of both Asp^{125} and Arg^{126} caused almost complete loss of the agonist-induced inositol phosphate response (Fig. 2A), despite the normal expression level of the DRY/AAY receptor (Table 1).

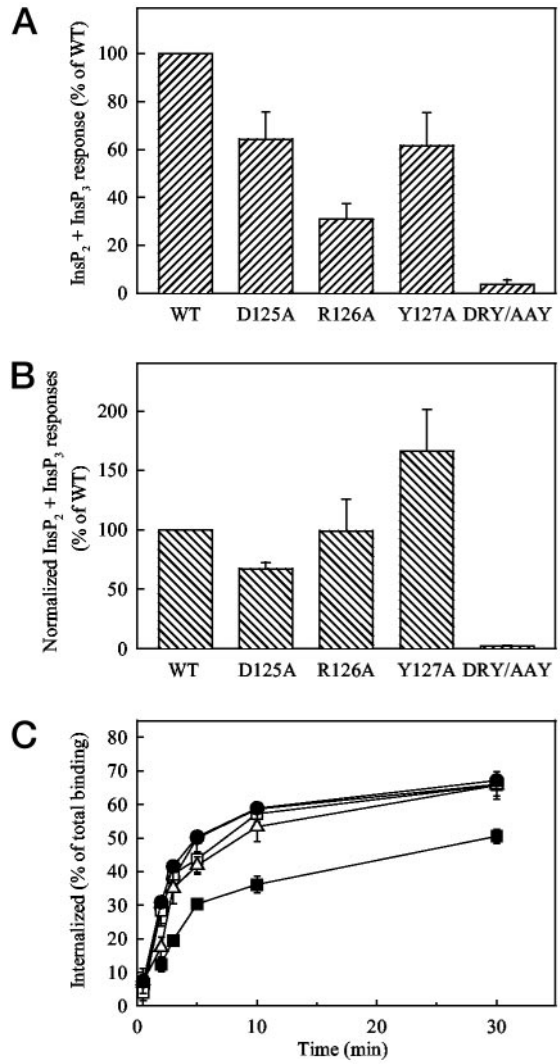

FIG. 2. Inositol phosphate responses and internalization kinetics of DRY-mutant AT_{1A} receptors. A, Transfected COS7 cells were prelabeled with [^{3}H]inositol, pretreated with LiCl, and incubated in the absence or presence of 1 μM Ang II as described in *Materials and Methods*. The combined $InsP_2$ + $InsP_3$ responses (stimulated minus basal) for each mutant are presented as percent of that of the wild-type receptor. B, The combined $InsP_2$ + $InsP_3$ responses are presented after normalization to the expression level (Table 1) of the corresponding receptors. Data are shown as percent of the normalized response of wild-type (WT) receptor. C, Internalization kinetics of the wild-type (●), D125A (△), R126A (○), Y127A (□), and DRY/DAA (■) AT_{1A} receptors were determined as described in *Materials and Methods*. Internalized radioactivity was expressed as percent of the total binding at each time point. All data are shown as means ± SEM from three independent experiments, each performed in duplicate.

TABLE 1. ^{125}I-[Sar^1,Ile^8]Ang II binding parameters for wild-type and mutant AT_{1A} receptors expressed in COS-7 cells

Mutant	K_d (nM)	Binding sites (% of WT)	n
WT	2.0 ± 0.3	100	10
D125A	2.6 ± 0.4	97 ± 18	7
R126A	1.8 ± 0.3	35 ± 11	4
Y127A	1.7 ± 0.3	45 ± 19	4
DRY/AAY	2.0 ± 0.4	89 ± 10	4
L128A	1.8 ± 0.3	82 ± 12	3
I130A	2.2 ± 0.2	74 ± 15	3
V131A	2.6 ± 0.3	75 ± 9	3
H132A	2.3 ± 0.2	81 ± 11	3
P133A	1.9 ± 0.1	91 ± 9	3
M134A	1.8 ± 0.2	91 ± 18	3
M134D	1.6 ± 0.2	45 ± 17	3

K_d and B_{max} values were calculated using the LIGAND program. The numbers of expressed binding sites are shown as a percent of the binding sites of the WT AT_{1A} receptor measured in the same experiment. The expression level of the WT AT_{1A} receptor was 2.40 ± 0.66 pmol/mg protein. Data are expressed as means ± SEM from the indicated number of independent experiments, each performed in duplicate.

Internalization kinetics of $D^{125}R^{126}Y^{127}$-mutant AT_{1A} receptors

The kinetics of dynamin- and β-arrestin-dependent internalization of the AT_{1A} receptor was determined by measuring the endocytosis of ^{125}I-Ang II (10, 39, 41). The internalization kinetics of the D125A, R126A, and Y127A mutants were similar to those of the wild-type AT_{1A} receptor (Fig. 2B), but that of the DRY/AAY mutant AT_{1A} receptor was significantly impaired. The endocytotic rate constants of the wild-type and DRY/AAY mutant AT_{1A} receptors in this series of experiments were 0.39 ± 0.04 and 0.13 ± 0.01 per min, respectively (n = 4).

Inositol phosphate responses of ^{128}LAIVHPM^{134} mutant AT_{1A} receptors

Charged residues in the carboxyl-terminal portion of the second intracellular loop of the AT_1 receptor have been implicated in G protein coupling (34, 35, 42). However, the amino-terminal region of IL-2, following the DRY sequence, contains a conserved sequence with several hydrophobic amino acids that has been suggested to be involved in the G protein coupling of several other GPCRs (23, 43). Also, histidine substitutions of the first four amino acids of this region in the AT_1 receptor were found to interfere with receptor activation (36). Triple alanine replacement of the conserved amino-terminal residues of IL-2 significantly decreased the inositol phosphate response of the mutant receptors, but only slight to moderate decreases were observed in the internalization kinetics of the receptors (data not shown). To identify single amino acids responsible for G protein coupling, alanine substitutions were made in the region of amino acids 128–134 (Fig. 1). Alanine replacements of amino acids 128–134 did not affect the binding affinity of the receptor for [Sar^1,Ile^8]AngII, and the expression levels of the receptors are shown in Table 1. In contrast, the inositol phosphate responses of these mutants were impaired to various extents, except for those bearing the L128A and V131A mutations, which had no effect on receptor function (Fig. 3A). The most

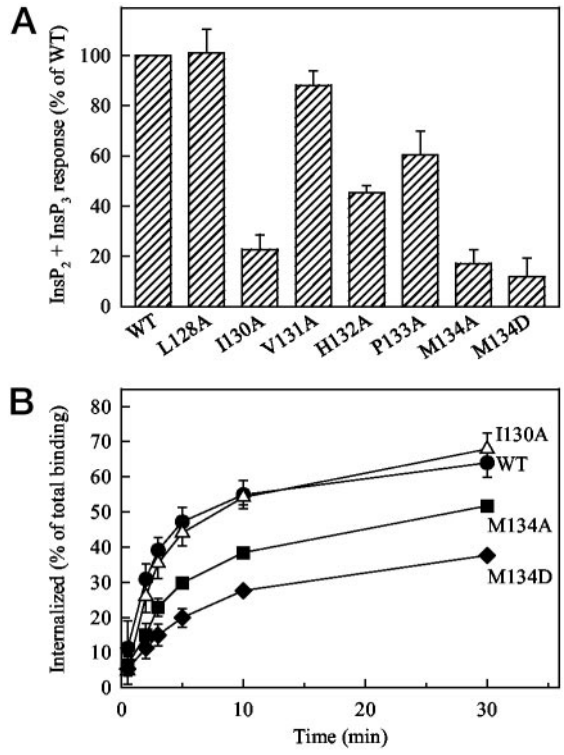

FIG. 3. Inositol phosphate responses and internalization kinetics of ^{128}LAIVHPM^{134} mutant AT_{1A} receptors. A, Transfected COS7 cells were prelabeled with [^{3}H]inositol. The cells were pretreated with LiCl and incubated in the absence or presence of 1 μM Ang II as described in *Materials and Methods*. Data are shown as percent of inositol phosphate response of the wild-type (WT) receptor. B, Internalization kinetics of the wild-type (●), I130A (△), M134A (■), and M134D (◆) mutant AT_{1A} receptors were determined as described in *Materials and Methods*. Internalized radioactivity was expressed as percent of the total binding at each time point. All data are shown as means ± SEM from three independent experiments, each performed in duplicate.

significant loss of function was caused by substitution of Ile^{130} and Met^{134} with Ala, suggesting that these amino acids have a role in G protein coupling. Met^{134} was replaced with aspartic acid to evaluate the importance of the neutrality of this position. The M134D mutation resulted in further impairment of the inositol phosphate response (Fig. 3A). This finding is consistent with the conservation of apolar amino acids or methionine in this position (13, 30–33, 44).

Internalization kinetics of ^{128}LAIVHPM^{134} mutant AT_{1A} receptors

Substitution of Met^{134} with Ala reduced the rate of internalization, and this was further decreased by its replacement with Asp. The I130A (Fig. 3B) and all other (data not shown) mutant receptors were internalized at the same rate as the wild-type receptor. Earlier studies have suggested that the structural requirements for internalization and signal generation of the AT_1 receptor are divergent. The present data indicate that this region of the IL-2 could be involved in direct interaction with the G protein, and that the methionine residue at position 134 may have a more complex role in receptor activation than the other nonpolar amino acids in this region.

ERK response of mutant receptors

The effect of alanine substitutions upon another intracellular signaling pathway, the extracellular signal regulated kinase pathway, was also studied. Although ERK activation by GPCRs may occur via β-arrestin-mediated mechanisms (45), several studies on the activation of ERK by the AT_{1A} receptor have suggested that nuclear targets of ERK are activated by pathways initiated by Ca^{2+} signal generation and protein kinase C (42, 46). However, the structural requirements in IL-3 for Ang II-induced ERK activation has been reported to differ from those for inositol phosphate signal generation (47). The role of the conserved DRYXXV/IXXPL sequence in ERK signaling of the receptor was evaluated by determining the ability of the mutant receptors to activate Elk1, a nuclear target of ERK activation. Activation of the Elk1 transcription factor was monitored by measuring luciferase activity in cells coexpressing the mutant AT_1 receptors with commercially available plasmids that encode the activation domain of Elk1 fused to the yeast GAL4 DNA binding domain, and firefly luciferase with a synthetic promoter containing five tandem repeats of the yeast GAL4-binding site. Ang II-induced luciferase activity was reduced by 74% in cells treated with the MEK1 inhibitor PD98059 (data not shown), suggesting the important role of ERK in activation of the Elk1 promoter. Ang II induced a 7.17 ± 1.31-fold increase of Elk1-mediated luciferase activity (Fig. 4), which was not inhibited significantly by pretreatment with pertussis toxin (16 h, 300 ng/ml, data not shown). Ang II-induced luciferase activity was similar to that of the wild-type receptor in case of L128A and P133A mutants and only slightly reduced in cells expressing the D125A, Y127A V131A mutant AT_{1A} receptor. However, Elk1-mediated luciferase activation was significantly impaired in all other alanine substitution-mutant AT_{1A} receptors (Fig. 4).

The amplitudes of mutant receptor ERK responses were well correlated with their inositol phosphate responses, as shown in Fig. 5. To determine whether the decreased ERK responses of the mutant receptors were caused by their impaired G protein coupling, we analyzed the relation between the amplitude of the ERK and inositol phosphate responses of the mutant receptors. As shown in Fig. 5, the Pearson's *r* value was 0.9 and the linear correlation between the ERK and $InsP_3$ responses of the mutant receptors was significant ($P < 0.05$). The only exception was the P133A mutant, which had a normal ERK response and a slightly reduced $InsP_3$ response, compared with the wild-type receptor. Because pertussis toxin has no significant inhibitory effect on Ang II-stimulated luciferase activity (data not shown), our results suggest that Elk activation in the nucleus via the ERK cascade is a consequence of the inositol phosphate response of the receptor. These findings are in agreement with recent studies suggesting that in COS7 cells, Ang II-induced ERK-mediated transcriptional activation via the AT_1 receptor is mediated by Ca^{2+} signal generation (42, 46).

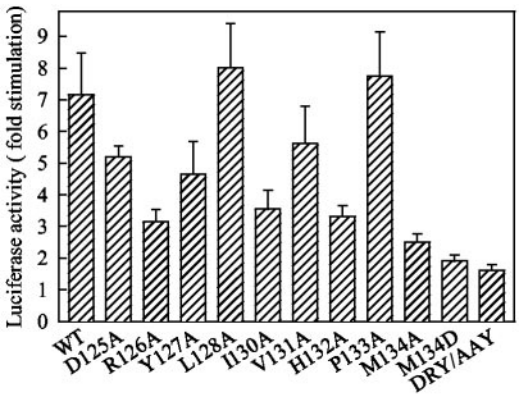

FIG. 4. ERK responses of the AT_{1A} receptor mutants. COS7 cells were transiently transfected with mutant or wild-type (WT) receptors and plasmids encoding the activation domain of Elk1 fused to the yeast GAL4 DNA binding domain and firefly luciferase with a promoter with yeast GAL4 binding sites. Cells were stimulated with 1 μM Ang II in serum-free DMEM for 5 h, and luciferase activity was determined in cell lysates. Data are shown as percent increase of luciferase activity divided by activity in control cells. All data are shown as means ± SEM from five experiments, each performed in duplicate.

Constitutive activity of the D125A mutant receptor

Previous studies on monoaminergic GPCRs detected constitutive receptor activity after mutating residues of the DRYXXV/IXXPL sequence (23, 29, 48). As detailed earlier, studies on monoaminergic receptors have suggested that the aspartic acid in the conserved DRY sequence is an important factor in maintaining the inactive state of the receptor, and mutations of this residue cause constitutive activation (14–19). In peptidergic GPCRs, the only evidence for enhanced inositol phosphate signal generation was observed in a mutant GnRH receptor (20). However, no increases in basal inositol phosphate levels were detected in the mutated AT_{1A} receptors in the present study, despite their relatively high expression levels. Nevertheless, the basal luciferase activities in cells expressing the D125A mutant were significantly elevated, compared with that of the wild-type receptor (Fig. 6A, n = 5, $P < 0.05$). Ang II-stimulated luciferase activity showed parallel increase with that of the basal level (Fig. 6A) without enhancement of the Ang II response (Fig. 4). To test the hypothesis that the slightly elevated luciferase activity of the D125A mutant was caused by moderate constitutive ac-

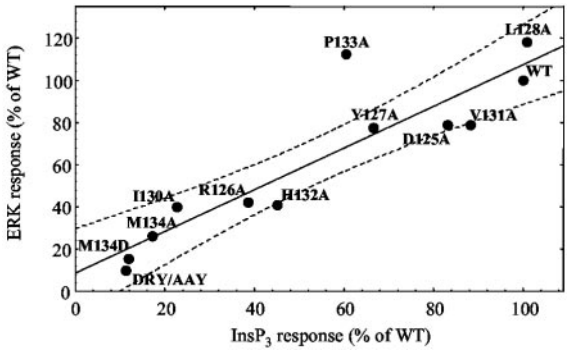

FIG. 5. Linear correlation between $InsP_3$ and ERK responses of mutant AT_1 receptors. $InsP_3$ and ERK responses of the mutant receptors are indicated as percent of the response of the wild-type (WT) receptor. Correlation between $InsP_3$ and ERK responses of the mutant receptors was analyzed by STATISTICA computer program. Pearson's correlation coefficient was 0.901. *Dotted lines* indicate the 95% confidence interval for the regression line.

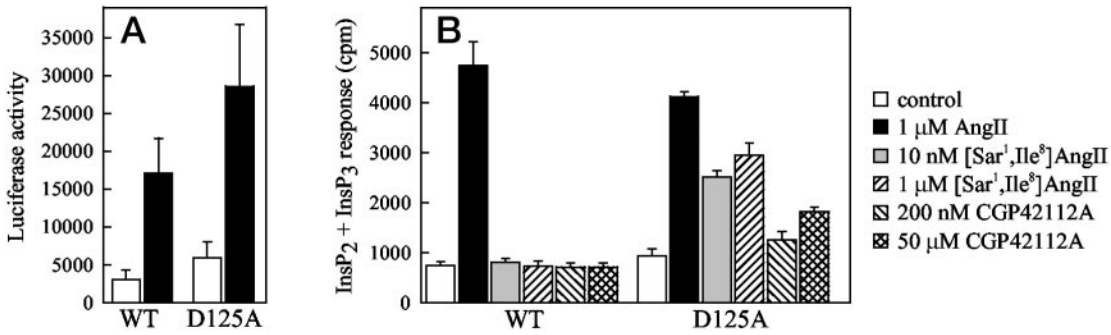

FIG. 6. ERK and inositol phosphate responses of D125A and wild-type AT_{1A} receptors. A, ERK responses in cells expressing D125A or wild-type (WT) AT_{1A} receptors. Luciferase activity was measured in cells expressing the D125A and wild-type AT_{1A} receptors and plasmids encoding the Elk1 fused to the yeast GAL4 DNA-binding domain and firefly luciferase with a promoter with yeast GAL4-binding sites. Then the cells were incubated in the absence or presence of 1 μM Ang II in serum-free DMEM for 5 h, and luciferase activity of cell lysates were determined. All data are shown as means ± SEM from five experiments, each performed in duplicate. B, Inositol phosphate responses of D125A and wild-type AT_{1A} receptors stimulated by AT_1 receptor partial agonists. Transfected COS7 cells were prelabeled with [^{3}H]inositol. The cells were pretreated with LiCl and incubated in the absence or presence of the indicated concentration of Ang II, [Sar^1,Ile^8]Ang II, and CGP42112A for 20 min as described in *Materials and Methods*. Radioactivities of the combined $InsP_2$ + $InsP_3$ fractions are shown as means ± SEM from three experiments, each performed in duplicate.

tivity, we analyzed the effects of partial agonists on inositol phosphate signaling of the D125A receptor. Previous studies have shown that constitutively activated AT_1 receptors exhibit markedly increased inositol phosphate responses to peptide partial agonists, such as CGP42112A and [Sar^1,Ile^8]Ang II (49, 50). Although these agents did not affect inositol phosphate levels in cells expressing the wild-type AT_{1A} receptor, both peptides stimulated inositol phosphate production in cells expressing the D125A mutant receptor (Fig. 6B). These data suggest that substitution of Asp^{125} with alanine results in an AT_{1A} receptor with moderate constitutive activity that is more readily detected in the basal level of Elk1-regulated luciferase expression than in the rate of inositol phosphate production.

Discussion

Agonist binding to GPCRs evokes a conformational change that leads to activation of the receptor and coupling to its intracellular signaling pathways. Although the exact mechanism of this change is not known, it probably involves rearrangements of the intramolecular interactions within the seven transmembrane domain structure of the receptor. The highly conserved DRY sequence at the C-terminal end of the third intramembrane helix is generally considered to be a major structural determinant of GPCR function (12, 23, 51). Although conservative substitutions are tolerated at the positions of the aspartic acid and tyrosine, the arginine residue in the center of this triplet is invariant (44, 52). Initial studies on mutant rhodopsins suggested that the arginine residue is required for binding of transducin (53, 54). However, a more recent study showed that R135G mutant rhodopsin binds the transducin (340–350) peptide and suggested that the role of the conserved aspartate/glutamate-arginine sequence is to induce GDP dissociation from transducin (14). In the *N*-formyl peptide receptor, mutations of the conserved aspartate and arginine residues prevented receptor binding to G proteins and completely eliminated ligand-induced Ca^{2+} signal generation (55). The importance of the conserved DRY sequence in AT_1 receptor activation was first demonstrated by Ohyama *et al.* (34), who also reported that the initial phase of inositol phosphate signal generation is markedly impaired in D125A or G and R126A or G receptors (35). The present study using more prolonged Ang II stimulation showed that single alanine substitutions of Asp^{125} and Arg^{126} modestly affect inositol phosphate signal generation by the AT_1 receptor, which was demonstrated by the increased EC_{50}-s of Ang II-induced inositol phosphate generation. These findings are consistent with partial impairment of the ligand-induced activation of these mutant receptors.

Aspartic acid or glutamic acid residues precede the conserved arginine residue in 99% of rhodopsin-like GPCRs (12, 23, 44, 52). The negative charge at this residue of the DRY sequence has been found to inhibit G protein activation of rhodopsin (14, 15). Increased basal signaling was also reported after substitution of the conserved aspartate in several aminergic receptors, including the α_{1B}- (16, 17), β_2- (18) adrenergic receptors, and the H2 histamine receptor (19). In accordance with these findings, it has been suggested that activation of GPCRs involves protonation of the conserved aspartate residue (16, 22, 23). The crystal structure of the inactive conformation of rhodopsin supports the proposed role of the conserved Glu/Asp residue in stabilizing the inactive receptor conformation by forming a salt bridge with the adjacent conserved arginine residue (11, 12). An interaction between this conserved arginine residue and a Glu (and Thr) in helix VI is also suggested in these studies, and it may represent one of the critical constraints keeping rhodopsin in the inactive conformation (11, 12).

Very recent studies suggest that negatively charged residues in IL-3 adjacent to helix VI may also participate in this process in the α_{1b}-adrenergic receptor (13, 56). In peptidergic receptors the conserved aspartate residue may similarly limit receptor activation because its substitution in the GnRH receptor causes increased agonist-induced receptor activation (20). However, other studies reported impaired receptor folding and failed to detect constitutive receptor activation after charge-neutralizing mutations of the conserved as-

partic acid residue in aminergic GPCRs (27, 29, 57). After charge-neutralizing mutations of the conserved Asp^{125} of the AT_{1A} receptor, the present study and others (34, 35, 42) revealed no elevation of inositol phosphate levels in unstimulated cells. However, we observed that constitutive activation of the receptor was detectable after stimulation with partial agonists. These data suggest that Asp^{125}, as in aminergic receptors, has a role in stabilizing the inactive conformation of the AT_1 receptor, and point to a similarity in the activation process of aminergic and peptidergic GPCRs. However, in the AT_1 receptor additional interactions, such as the previously proposed interaction of Tyr^{292} and Asn^{111} (58), may have equally important roles in this process. For this reason, elimination of the charge of Asp^{125} is not sufficient to induce detectable signal generation in the absence of agonists. In addition, several other studies on α_{2A}-adrenergic, β_2-adrenergic, and M1 muscarinic receptors have shown that G protein activation is also impaired by mutations of the conserved aspartic acid residue (51). In the present study, substitution of Asp^{125} and Arg^{126} with alanine cause additive impairment of inositol phosphate signal generation, indicating that the conserved aspartate residue also has a function in the stabilization of the active conformation of the receptor.

Mutations of the conserved arginine residue abolish signaling of the M1 muscarinic receptor (25, 26, 43, 57) and other GPCRs (16, 20, 24, 27). However, site-directed mutagenesis of several GPCRs has demonstrated that the presence of this residue is not an absolute requirement for receptor activation. Thus, point mutations of the conserved arginine residue in the M_2 muscarinic receptor (26) and GnRH receptor (20) reduced adenylyl cyclase inhibition and inositol phosphate generation, respectively, but did not completely abolish agonist-induced signaling by these receptors. The present study shows that in the AT_1 receptor, a peptidergic GPCR, substitution of Arg^{126} with alanine causes a modest impairment of inositol phosphate signal generation, and double alanine substitutions of Asp^{125} and Arg^{126} were required to cause major impairment of signal generation. Earlier studies detected more complete inhibition of Ang II-induced inositol phosphate signal generation at very early time points (35), which is consistent with reports on other mutations that cause partial inhibition of G protein activation and possibly reflects the delayed kinetics of signal generation in these mutants (40, 59–61). However, it has been suggested recently that substitution of the arginine residue in the conserved DRY sequence causes constitutive desensitization and agonist-independent β-arrestin binding of the AT_1 receptor and other GPCRs (62).

These findings may explain the partially impaired inositol phosphate responses of the R126A mutant receptor observed in the present study. Inositol phosphate signal generation was more seriously impaired after replacement of both Asp^{125} and Arg^{126} residues with alanine in the DRY/AAY mutant AT_{1A} receptor. These data suggest that the aspartate and arginine residues in the DRY sequence of the AT_1 receptor have a complementary function in an event required for G protein activation. Studies on rhodopsin have shown that the third and sixth helices are separated during activation, exposing the conserved DRY sequence (63). The internalization kinetics of the DRY/AAY mutant AT_{1A} receptor were also impaired, albeit less severely than inositol phosphate signaling. A similar parallel impairment of inositol phosphate signaling and internalization kinetics was observed after replacing Arg^{139} (corresponding to Arg^{126}) in the AT_{1A} receptor) of the GnRH receptor (20). Because inositol phosphate signal generation and agonist-induced internalization of the AT_1 receptor have different structural requirements (37, 64), the simplest explanation of such parallel impairment of signaling and internalization pathways is that amino acids of the conserved DRY sequence have a structural role in the agonist-induced conformational change during receptor activation.

The tyrosine residue in the DRY sequence is the least conserved residue in this triplet and is found in 75% of rhodopsin-like GPCRs (52). Mutations of this amino acid caused less marked changes in GPCR function. In studies on the M1 muscarinic receptor (26) and rhodopsin (65), point mutations of this residue had inhibitory effects on signaling and possibly on receptor processing. In the GnRH receptor, the tyrosine residue is normally replaced by serine, but reinsertion of the tyrosine at this position caused no major functional change (20). Mutation of Tyr^{127} in the AT_{1A} receptor likewise had no substantial functional consequences.

The amino acids of IL-2 C-terminal to the conserved DRY sequence were found to serve as a switch that enables G protein coupling in the M5 muscarinic receptor (29). In this report, mutations on one side of this helical region cause constitutive activation, whereas residues on the other side of the helix are required for receptor activation (29). The present study shows that the same surface of the AT_{1A} receptor is also required for receptor activation. It is particularly notable that Ile^{130} on this surface is selectively required for inositol phosphate signal generation but has no role in agonist-induced internalization. These findings make Ile^{130} a potential candidate for direct interaction with G_q. The role of this apolar surface of IL-2 in agonist-induced signal generation is consistent with results obtained with other GPCRs, including the M1, M3, and M5 muscarinic; GnRH; V_{1A} vasopressin; and PTH/PTHRP receptors, all of which couple to G_q (29–31, 43, 66, 67). The Met^{134} residue is also strongly required for agonist-induced signal generation. A similar functional role of the conserved apolar residue (usually leucine) at the C-terminal end of the DRYXXV/IXXPL sequence has been reported for several other GPCRs (13, 30–33). However, our finding that Met^{134} of the AT_{1A} receptor is also required for normal agonist-induced internalization suggests that this residue may have a complex role during receptor activation.

Ang II-induced Elk1-mediated luciferase response, which reflects mainly the agonist-induced ERK activation, showed good correlation with the inositol phosphate responses. These data, and the pertussis toxin insensitivity of the luciferase response, suggest that AT_1 receptor-mediated ERK activation is mainly G_q mediated in COS7 cells. Thus, the ERK activation pathway in these cells is more similar to that in vascular smooth muscle cells (2) and different from the G_i-mediated mechanism observed in adrenal glomerulosa cells (68).

In conclusion, this study has shown that although activation of the AT_1 angiotensin receptor requires the presence of

the conserved aspartic acid and arginine residues of the DRY triplet, individual substitution of these amino acids has no major effect on receptor function. Substitution of Asp^{125} in this sequence with alanine causes constitutive activation, which can be detected with partial agonists that are unable to stimulate inositol phosphate production in cells expressing the wild-type receptor. In the extension of TM3 distal to the conserved DRY sequence, an apolar surface that includes Ile^{130} and Met^{134} appears to have a direct role in agonist-induced coupling of the AT_{1A} receptor to G_q.

Acknowledgments

We thank Dr. H. S. Wiley for providing the algorithms for the calculation of the endocytotic rate constant. The excellent technical assistance of Judit Bakacsiné Rácz, Katinka Süpeki, and Yue Zheng are greatly appreciated.

Received December 12, 2002. Accepted March 3, 2003.

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This work was supported in part by grants from the Hungarian Ministry of Public Health (ETT 315/2000), Hungarian Science Foundation (OTKA T-032179 and OTKA Ts-040865), and Hungarian Ministry of Education (FKFP 0318/1999).

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